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Term:	L3 and block copolymer
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<u>L3</u>	L2 and adding protein	59	<u>L3</u>
<u>L2</u>	L1 and polymer same protein	5521	<u>L2</u>
<u>L1</u>	membrane and copolymer and protein	12209	<u>L1</u>

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Term:	L2 and adding protein
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<u>L3</u>	L2 and adding protein	59	<u>L3</u>
<u>L2</u>	L1 and polymer same protein	5521	<u>L2</u>
<u>L1</u>	membrane and copolymer and protein	12209	<u>L1</u>

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☐ 1. Document ID: US 5998588 A

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File: USPT

Dec 7, 1999

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DOCUMENT-IDENTIFIER: US 5998588 A

**** See image for Certificate of Correction ****

TITLE: Interactive molecular conjugates

DATE-ISSUED: December 7, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Hoffman; Allan S.	Seattle	WA		
Stayton; Patrick S.	Seattle	WA		

US-CL-CURRENT: 530/402; 424/178.1, 424/193.1, 424/280.1, 424/78.08, 424/94.1,
530/350, 530/387.1, 530/391.1, 530/395, 530/399, 530/403, 530/404, 530/405, 530/406

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L5: Entry 1 of 1

File: USPT

Dec 7, 1999

DOCUMENT-IDENTIFIER: US 5998588 A

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TITLE: Interactive molecular conjugates

Abstract Text (1):

→ The combination of the capabilities of stimuli-responsive components such as polymers and interactive molecules to form site-specific conjugates which are useful in a variety of assays, separations, processing, and other uses is disclosed. The polymer chain conformation and volume can be manipulated through alteration in pH, temperature, light, or other stimuli. The interactive molecules can be biomolecules like proteins or peptides, such as antibodies, receptors, or enzymes, polysaccharides or glycoproteins which specifically bind to ligands, or nucleic acids such as antisense, ribozymes, and aptamers, or ligands for organic or inorganic molecules in the environment or manufacturing processes. The stimuli-responsive polymers are coupled to the recognition biomolecules at a specific site so that the polymer can be manipulated by stimulation to alter ligand-biomolecule binding at an adjacent binding site, for example, the biotin binding site of streptavidin, the antigen-binding site of an antibody or the active, substrate-binding site of an enzyme. Binding may be completely blocked (i.e., the conjugate acts as an on-off switch) or partially blocked (i.e., the conjugate acts as a rheostat to partially block binding or to block binding only of larger ligands). Once a ligand is bound, it may also be ejected from the binding site by stimulating one (or more) conjugated polymers to cause ejection of the ligand and whatever is attached to it. Alternatively, selective partitioning, phase separation or precipitation of the polymer-conjugated biomolecule can be achieved through exposure of the stimulus-responsive component to an appropriate environmental stimulus.

Brief Summary Text (3):

Protein conjugates with other molecules have long been used for protection of the protein from recognition, e.g., PEGylated proteins, targeting purposes and in separations and diagnostics technology, for example, streptavidin reactions and antibody-mediated affinity chromatography and antibody-enzyme conjugates, as in ELISA. Most of these are examples of non-site-specific conjugates, such as PEGylated proteins, antibody-drug bioconjugates and polymer-drug conjugates.

Brief Summary Text (4):

Polymers and small molecules have also been site-specifically conjugated to proteins for other purposes. For example, as reported by Chilkoti, et al. (1993), a stimuli-responsive polymer was site-specifically conjugated to a genetically engineered protein in order to precisely control the polymer:protein stoichiometry and to locate the polymer away from the protein active site, so that a physical phase separation could be carried out without interfering with the recognition site binding activity. Site-specific conjugation of polyethylene glycol to proteins via genetically-engineered cysteine residues has also recently been reported by Goodson and Kaktre, 1990; and Benhar, et al. 1994). These studies were designed to increase the circulation time and stability of therapeutic proteins without interfering with their activity, by assuring that the conjugated polymer was attached away from the

active site. Polylysine polymers have also been conjugated to antibody fragments via existing thiol groups, in order to achieve better biodistribution and greater bioactivity of imaging and therapeutic antibodies, as reported by Slinkin, et al. 1992. Numerous examples of engineering cysteine residues for site-specific conjugation of small molecules, such as fluorophores or drugs, have been described by Stayton, et al. 1988). The photoswitchable binding of ligands to some protein binding sites via photochromic dye conjugation has also been reported by Willner and Rubin 1992; Willner et al. 1993. A photochromic dye that exists as two light-interconvertible isomers was conjugated randomly to protein lysine amino sites. Protein activity was shown to be dependent on the state of isomerization, so the protein could be switched between active and inactive forms by the photoisomerization of the dye. Concanavalin A binding to saccharides has been controlled by non-specific conjugation of several thiophenfulgide dyes to lysine residues (Willner and Rubin, 1992). The photoregulated activity of acetylcholinesterase and chymotrypsin via photochromic enzyme inhibitors has also been demonstrated by Bieth, et al. 1969.

Brief Summary Text (5):

Despite the recognition capabilities of proteins, technology based on protein catalysis, separations or delivery is often burdened by materials and processing costs. These drawbacks are related to the need to control several fundamental molecular processes such as protein recognition and binding to the target molecule, reaction kinetics, and separation and purification of the target molecule or its reaction by-product.

Brief Summary Text (6):

Current methods for controlling the sequential binding and release of target ligands in affinity-based technologies generally depend on the immobilization of the affinity agent on a solid surface, contact with a solution containing the target ligand and other species, to allow specific binding of the ligand to the affinity agent, and, for elution of a target ligand, the use of chemicals which are potential denaturants and/or solutions with large changes in pH or ionic strength. Processing steps are generally slow, requiring lengthy equilibration with large volumes of expensive buffer mixtures, and thus are often a primary hurdle in cost-effective separations. Non-specific binding of non-target molecules often contaminate the product. The need for advanced elution strategies in affinity-based techniques has been noted in the literature. (Yarmush et al., Biotechnol. Prog. 8:168-178 (1992)). Similarly, biosensor technologies (e.g., environmental or biological stream analysis) employing a protein-ligand detection system require elution of the bound target to regenerate the biosensor. Current immunoadsorbent, biosensor and chromatographic processing (e.g., elution) strategies would greatly benefit from a controlled, triggered release step providing faster response times and cleaner, purer and more concentrated separation streams.

Brief Summary Text (11):

The combination of the capabilities of stimulus-responsive components and interactive molecules to form site-specific conjugates which are useful in a variety of assays, separations, processing, and other uses is disclosed. The polymers can be manipulated through alteration in pH, temperature, light, or other stimuli. The interactive molecules can be a biomolecule, such as (a) peptides or proteins, for example, antibodies, receptors, or enzymes, (b) polysaccharides or glycoproteins, or (c) nucleic acids such as antisense, ribozymes, and aptamers, all of which specifically bind to ligands or receptors, or a ligand for an organic or inorganic compound, for example, a metal chelating agent. The stimuli-responsive compounds are coupled to the interactive molecules at a specific site so that the stimulus-responsive component can be manipulated to alter ligand binding at an adjacent ligand binding site, for example, the antigen-binding site of an antibody or the active site of an enzyme. Binding may be completely blocked (i.e., the conjugate acts as an on-off switch) or partially blocked (i.e., the conjugate acts as a rheostat to partially block binding). Partial blocking can be used to effect

selective binding, by still allowing small ligands to bind but totally blocking larger ligands.

Brief Summary Text (12):

These conjugates provide precise control of molecular interactions, for example, protein recognition steps or enzyme reaction steps in the processes by external signals, which avoid undesirable interferences or damage to the interactive molecules and targets during affinity binding, reaction (catalysis), or "unbinding" (separation). The interactive molecular conjugate systems are easy to adapt to a wide variety of separation, diagnostic, reaction, enzyme processes and delivery applications, and are economical to use.

Brief Summary Text (13):

Examples demonstrate formation of a site-specific conjugate by genetically engineering a protein, streptavidin, to insert a coupling site, then coupling a T-responsive polymer, polyNIPAAm, to the coupling site. The physical relationship (conformation) of the polymer to the biotin binding site of the streptavidin is controlled by altering the temperature of the reaction; i.e., at low temperatures, 100% of the biotin is bound, at higher temperatures, 37.degree. C., significantly less biotin is bound.

Drawing Description Text (2):

FIG. 1a is a schematic illustration of a stimulus-responsive polymer, site-specifically conjugated to a protein, that functions as a ligand affinity switch.

Drawing Description Text (3):

FIG. 1b is a schematic illustration of graft and block copolymers and random copolymers which enhance the stimulus response.

Drawing Description Text (7):

FIGS. 3a-c are schematic illustrations of an externally-triggered and reverse engineered polymer-protein conjugate designed for surface or interfacial or two phase partitioning separations. FIG. 3a shows the stimulus-interactive polymer in an unfolded or relaxed state. FIG. 3b shows the stimulus-interactive polymer in a collapsed form after exposure to a stimulus. FIG. 3c shows the same conjugate but with the stimulus-interactive polymer in an intermediate state between the uncollapsed or relaxed form of FIG. 3a and the completely collapsed form of FIG. 3b.

Drawing Description Text (9):

FIG. 5 is a schematic illustration of the use of a responsive-polymer/engineered-protein conjugate in a separations application. The support can be a conventional chromatographic resin, an HPLC support, a particulate or porous support such as a microporous membrane or hollow fiber, a bioreactor surface, or other means for immobilization. As depicted, the receptor is streptavidin having conjugated thereto a stimulus-responsive polymer and the ligand is a biotinylated protein such as a biotinylated antibody, or enzyme, which further includes a binding site for an analyte or other target molecule and a stimulus-responsive molecule which can block or allow binding upon exposure to a second, different stimulus. The same format can be used in aqueous two-phase separations, where the polymer-protein conjugates are reversibly partitioned in one or the other phase, or at the interface between them.

Drawing Description Text (11):

FIG. 7 illustrates an embodiment of the conjugated interactive molecule for the targeted delivery of drugs from a streptavidin delivery protein which is complexed both by a biotinylated-monoclonal antibody and by a biotinylated-drug. The biotinylated-drug binding sites are also conjugated with stimulus-responsive polymer, so that when the polymer is triggered by an external stimulus (e.g., a light pulse) the biotinylated drug is ejected from the binding pocket and delivered

to the target cell. The two polymers may be triggered by the same or by different stimuli.

Drawing Description Text (15):

FIGS. 11a-c show the conjugate of a responsive copolymer and an enzyme, where the responsive copolymer has pendant drug molecules conjugated to the copolymer chain backbone by linkages that are degradable by the same enzyme to which the copolymer is conjugated. When the polymer is stimulated to collapse its coil, the enzyme is turned off, and it cannot release the drug by enzymolysis of the pendant linkages; when the reverse stimulus is applied, the enzyme becomes active and releases drug molecules by enzymolysis of the pendant linkages. A hydrogel, with an enzyme entrapped within the gel is shown in FIG. 11d.

Drawing Description Text (16):

FIGS. 12a and 12b are illustrations of ssDNA hooks for "conjugating" the responsive polymers to the protein.

Drawing Description Text (20):

FIG. 16 is a schematic illustration of the use of a conjugate of a stimulus-interactive molecule and an interactive molecule such as an enzyme, antibody, or receptor protein as an environmental sensor. The sensor protein is protected and inactive until exposed to a stimulus. The interactive molecule binds to a molecule such as an analyte, toxin, inhibitor or fouling agent. The sensor is regenerated by removal of the stimulus (or, not shown, exposure of a second stimulus-interactive molecule to a second stimulus).

Detailed Description Text (4):

FIG. 1A is a schematic of a conjugate 10 of a stimulus-interactive molecule 16 bound to protein 14, where the stimulus-interactive molecule 16 blocks the binding site 12 of the protein 14 when not exposed to the stimulus and does not block the binding site 12 upon exposure to a stimulus, or vice versa. When the binding site 12 is not blocked, a ligand 18 can bind to the protein 14.

Detailed Description Text (6):

FIG. 1B are representative forms of the stimulus-interactive molecule 16 of FIG. 1A, including linear homo- or co-polymers, grafts of comb or block copolymers, and star polymers.

Detailed Description Text (7):

FIG. 1C is a schematic of an alternative embodiment, where the stimulus-responsive molecule 16 is bound to the ligand 18, which in the presence of a stimulus is blocked from binding to the binding site 12 of the protein 14, or vice versa.

Detailed Description Text (8):

FIG. 1D is a schematic of yet another embodiment, which is essentially a combination of the embodiments of FIGS. 1A and 1C. The protein 14 includes a stimulus-responsive polymer 20 which, upon exposure to a first stimulus, blocks the binding site 12. The ligand 18 is also conjugated to a stimulus-responsive polymer 16, which upon exposure to a second, different stimulus, blocks binding of the ligand 18 to the binding site 12. This provides a means for varying binding affinity as well as acting to prevent or allow binding.

Detailed Description Text (11):

Stimulus-responsive components useful to make the conjugates described herein can be any which are sensitive to a stimulus that cause significant conformational changes in the polymer coils, and which can be engineered to contain a group which is reactive with specific groups (e.g., ligands) on the interactive molecule of interest. Illustrative polymers described herein are temperature-, pH-, ion- and/or light-sensitive polymers. Hoffman, A. S., "Intelligent Polymers in Medicine and Biotechnology", Artif. Organs, 19, 458-467 (1995); Chen, G. H. and A. S. Hoffman,

"A new temperature- and pH-responsive copolymer for possible use in protein conjugation", Macromol. Chem. Phys., 196, 1251-1259 (1995); Irie, M. and D. Kungwachakun, "Photoresponsive Polymers. Mechanochemistry of Polyacrylamide Gels having Triphenylmethane Leuco Derivatives", Makromol. Chem., Rapid Commun., 5, 829-832 (1985); and Irie, M., "Light-induced Reversible Conformational Changes of Polymers in Solution and Gel Phase", ACS Polym. Preprints, 27(2), 342-343 (1986); which are incorporated by reference herein.

Detailed Description Text (12):

Stimuli-responsive oligomers and polymers useful in the conjugates described herein can be synthesized that range in molecular weight from about 1,000 to 30,000 Daltons, with a reactive group at one or both chain ends. In a preferred embodiment, these syntheses are based on the chain transfer-initiated free radical polymerization of vinyl-type monomers, as described herein, and by (1) Tanaka, T., "Gels", Sci. Amer., 244, 124-138 (1981); 2) Osada, Y. and S. B. Ross-Murphy, "Intelligent Gels", Sci. Amer., 268, 82-87 (1993); (3) Hoffman, A. S., "Intelligent Polymers in Medicine and Biotechnology", Artif. Organs, 19, 458-467 (1995); also Macromol. Symp., 98, 645-664 (1995); (4) Feijen, J., I. Feil, F. J. van der Gaag, Y. H. Bae and S. W. Kim, "Thermosensitive Polymers and Hydrogels Based on N-isopropylacrylamide", 11th European Conf. on Biomtls., 256-260 (1994); (5) Monji, N. and A. S. Hoffman, "A Novel Immunoassay System and Bioseparation Process Based on Thermal Phase Separating Polymers", Appl. Biochem. and Biotech., 14, 107-120 (1987); (6) Fujimura, M., T. Mori and T. Tosa, "Preparation and Properties of Soluble-Insoluble Immobilized Proteases", Biotech. Bioeng., 29, 747-752 (1987); (7) Nguyen, A. L. and J. H. T. Luong, "Synthesis and Applications of Water-Soluble Reactive Polymers for Purification and Immobilization of Biomolecules", Biotech. Bioeng., 34, 1186-1190 (1989); (8) Taniguchi, M., M. Kobayahi and M. Fujii, "Properties of a Reversible Soluble-Insoluble Cellulase and Its Application to Repeated Hydrolysis of Crystalline Cellulose", Biotech. Bioeng., 34, 1092-1097 (1989); (9) Monji, N., C-A. Cole, M. Tam, L. Goldstein, R. C. Nowinski and A. S. Hoffman, "Application of a Thermally-Reversible Polymer-Antibody Conjugate in a Novel Membrane-Based Immunoassay", Biochem. and Biophys. Res. Comm., 172, 652-660 (1990); (10) Monji, N. C. A. Cole, and A. S. Hoffman, "Activated, N-Substituted Acrylamide Polymers for Antibody Coupling: Application to a Novel Membrane-Based Immunoassay", J. Biomtls. Sci. Polymer Ed., 5, 407-420 (1994); (11) Chen, J. P. and A. S. Hoffman, "Polymer-Protein Conjugates: Affinity Precipitation of Human IgG by Poly(N-Isopropyl Acrylamide)-Protein A Conjugates", Biomtls., 11, 631-634 (1990); (12) Park, T. G. and A. S. Hoffman, "Synthesis and Characterization of a Soluble, Temperature-Sensitive Polymer-Conjugated Enzyme, J. Biomtls. Sci. Polymer Ed., 4, 493-504 (1993); (13) Chen, G. H., and A. S. Hoffman, Preparation and Properties of Thermo-Reversible, Phase-Separating Enzyme-Oligo(NIPAAm) Conjugates", Bioconj. Chem., 4, 509-514 (1993); (14) Ding, Z. L., G. H. Chen, and A. S. Hoffman, "Synthesis and Purification of Thermally-Sensitive Oligomer-Enzyme Conjugates of Poly(NIPAAm)-Trypsin", Bioconj. Chem., 7, 121-125 (1995); (15) Chen, G. H. and A. S. Hoffman, "A New Temperature- and pH-Responsive Copolymer for Possible Use in Protein Conjugation", Macromol. Chem. Phys., 196, 1251-1259 (1995); (16) Takei, Y. G., T. Aoki, K. Sanui, N. Ogata, T. Okano, and Y. Sakurai, "Temperature-responsive Bioconjugates. 1. Synthesis of Temperature-Responsive Oligomers with Reactive End Groups and their Coupling to Biomolecules", Bioconj. Chem., 4, 42-46 (1993); (17) Takei, Y. G., T. Aoki, K. Sanui, N. Ogata, T. Okano and Y. Sakurai, "Temperature-responsive Bioconjugates. 2. Molecular Design for Temperature-modulated Bioseparations", Bioconj. Chem., 4, 341-346 (1993); (18) Takei, Y. G., M. Matsukata, T. Aoki, K. Sanui, N. Ogata, A. Kikuchi, Y. Sakurai and T. Okano, "Temperature-responsive Bioconjugates. 3. Antibody-Poly(N-isopropylacrylamide) Conjugates for Temperature-Modulated Precipitations and Affinity Bioseparations", Bioconj. Chem., 5, 577-582 (1994); (19) Matsukata, M., Y. Takei, T. Aoki, K. Sanui, N. Ogata, Y. Sakurai and T. Okano, "Temperature Modulated Solubility-Activity Alterations for Poly(N-Isopropylacrylamide)-Lipase Conjugates", J. Biochem., 116, 682-686 (1994); (20) Chilkoti, A., G. H. Chen, P. S. Stayton and A. S. Hoffman, "Site-Specific Conjugation of a Temperature-Sensitive Polymer to a Genetically-

Engineered Protein", Bioconj. Chem., 5, 504-507 (1994); and (21) Stayton, P. S., T. Shimoboji, C. Long, A. Chilkoti, G. Chen, J. M. Harris and A. S. Hoffman, "Control of Protein-Ligand Recognition Using a Stimuli-Responsive Polymer", Nature, 378, 472-474 (1995).

Detailed Description Text (13):

These types of monomers allow the design of copolymer compositions to respond to a specific stimulus and, in some embodiments, to two or more stimuli. In addition, control of molecular weight (by control of reactant concentrations and reaction conditions), composition, structure (e.g., linear homopolymer, linear copolymer, block or graft copolymer, "comb" polymers and "star" polymers, all of which may be incorporated into gel structure) and type and number of reactant end groups permit "tailoring" of the appropriate polymer for conjugation to a specific site on the interactive molecule. A genetically engineered temperature-sensitive peptide sequence may be constructed at the terminal end of the protein near or within the binding site. Peptide sequences which are pH, light, temperature, or chemical sensitive have also been reported and may be used to control the molecular recognition step via conjugation to the receptor or ligand, or through genetic incorporation into protein receptors or enzymes.

Detailed Description Text (15):

Illustrative embodiments of the many different types of temperature-responsive polymers which may be conjugated to interactive molecules are polymers and copolymers of N-isopropyl acrylamide (NIPAAm). PolyNIPAAm is a thermally sensitive polymer that precipitates out of water at 32.degree. C., which is its lower critical solution temperature (LCST), or cloud point (Heskins and Guillet, J. Macromol. Sci.-Chem. A2:1441-1455 (1968)). When polyNIPAAm is copolymerized with more hydrophilic comonomers such as acrylamide, the LCST is raised. The opposite occurs when it is copolymerized with more hydrophobic comonomers, such as N-t-butyl acrylamide. Copolymers of NIPAAm with more hydrophilic monomers, such as AAm, have a higher LCST, and a broader temperature range of precipitation, while copolymers with more hydrophobic monomers, such as N-t-butyl acrylamide, have a lower LCST and usually are more likely to retain the sharp transition characteristic of PNIPAAm (Taylor and Cerankowski, J. Polymer Sci. 13:2551-2570 (1975); Priest et al., ACS Symposium Series 350:255-264 (1987); and Heskins and Guillet, J. Macromol. Sci.-Chem. A2:1441-1455 (1968), the disclosures of which are incorporated herein). Copolymers can be produced having higher or lower LCSTs and a broader temperature range of precipitation.

Detailed Description Text (16):

Stimuli-responsive polymers such as poly(NIPAAm) have been conjugated randomly to affinity molecules, such as monoclonal antibodies, for example, as described in U.S. Pat. No. 4,780,409; Monji and Hoffman, Appl. Biochem. Biotechnol. 14:107-120 (1987). Activated groups (e.g. for conjugating to proteins), were formed randomly along the backbone of PNIPAAm and were conjugated randomly to lysine amino groups on a monoclonal antibody and the conjugate was then applied in a temperature-induced phase-separation immunoassay. Activated PNIPAAm has also been conjugated by Hoffman and coworkers to protein A, various enzymes, biotin, phospholipids, RGD peptide sequences, and other interactive molecules. The random polymer-interactive molecular conjugates have been used in a variety of applications based on the thermally-induced phase separation step (Chen and Hoffman, Biomaterials 11:631-634 (1990); Miura et al., Abstr. 17th Ann. Meet. Soc. Biomaterials (1991); Wu et al., Polymer 33:4659-4662 (1992); Chen and Hoffman, Bioconjugate Chem. 4:509-514 (1993); Morris et al., J. Anal. Biochem. 41:991-997 (1993); Park and Hoffman, J. Biomaterials Sci. Polymer Ed. 4:493-504 (1993); Chen and Hoffman, J. Biomaterials Sci. Polymer Ed. 5:371-382 (1994)). Others have also randomly conjugated proteins to PNIPAAm (Nguyen and Luong, Biotech. Bioeng. 34:1186-1190 (1989); Takei et al., Bioconj. Chem. 4:42-46 (1993)) and to pH-sensitive polymers (Fujimura et al., supra.)). Most of these polymer-protein conjugates involved random lysine amino groups of proteins bound to the polymer through random activated groups pendant

along the polymer backbone. More recently, a new method based on chain transfer initiation polymerization has been used which yields relatively low MW polymers (called oligomers) usually with only one reactive end group (but the method may be adapted to synthesis of oligomers with a reactive group at each end) (Otsu, T., et al. (1992) Eur. Polym. J., 28, 1325-1329). (Chen and Hoffman, 1993, supra; Chen and Hoffman, 1994, supra, and Takei et al., supra). The synthesis of an amino-terminated polymer proceeds by the radical polymerization of NIPAAm in the presence of AIBN as an initiator and 1-aminoethanethiol-hydrochloride as a chain transfer reagent. To synthesize a chain with --COOH or --OH terminal groups, carboxyl- or hydroxyl-thiol chain transfer agents, respectively, have been used instead of the amino-thiol. It should be noted that the synthesis of the end-reactive polymers is based on a chain transfer initiation and termination mechanism. This yields a relatively short polymer chain, having a molecular weight somewhere between 1000 and 25,000 to 30,000. The shortest chains, less than 10,000 in molecular weight, are usually called "oligomers". Oligomers of different molecular weights can be synthesized by simply changing the ratio of monomer to chain transfer reagent, and controlling their concentration levels, along with that of the initiator.

Detailed Description Text (19):

The molecular weight of vinyl-type copolymers can be controlled by varying the concentration of the key reactants and the polymerization conditions. However, it is difficult to achieve molecular weights much above about 30 kD using synthesis of vinyl-based oligomers by chain transfer initiation. Further, since the amino-thiol chain transfer agent yields a broader molecular weight distribution than the hydroxyl or carboxyl thiols (which may be undesirable), the --COOH-terminated polymer can be synthesized and the --COOH end group converted to an amine group by activating with carbodiimide and coupling a diamine to the active ester group.

Detailed Description Text (22):

Synthetic pH-sensitive polymers useful in making the conjugates described herein are typically based on pH-sensitive vinyl monomers, such as acrylic acid (AAc), methacrylic acid (MAAc), maleic anhydride (MANh), maleic acid (MAC), AMPS (2-Acrylamido-2-Methyl-1-Propanesulfonic Acid), N-vinyl formamide (NVA), N-vinyl acetamide (NVA) (the last two may be hydrolysed to polyvinylamine after polymerization), aminoethyl methacrylate (AEMA), phosphoryl ethyl acrylate (PEA) or methacrylate (PEMA). PH-sensitive polymers may also be synthesized as polypeptides from amino acids (e.g., polylysine or polyglutamic acid) or derived from naturally-occurring polymers such as proteins (e.g., lysozyme, albumin, casein, etc.), or polysaccharides (e.g., alginic acid, hyaluronic acid, carrageenan, chitosan, carboxymethyl cellulose, etc.) or nucleic acids, such as DNA. pH-responsive polymers usually contain pendant pH-sensitive groups such as --OPO(OH).sub.2, --COOH or --NH.sub.2 groups. With pH-responsive polymers, small changes in pH can stimulate phase-separation, similar to the effect of temperature on solutions of PNIPAAm (Fujimura et al. Biotech. Bioeng. 29:747-752 (1987)). By randomly copolymerizing a thermally-sensitive NIPAAm with a small amount (e.g. less than 10 mole percent) of a pH-sensitive comonomer such as AAc, a copolymer will display both temperature and pH sensitivity. Its LCST will be almost unaffected, sometimes even lowered a few degrees, at pHs where the comonomer is not ionized, but it will be dramatically raised if the pH-sensitive groups are ionized. When the pH-sensitive monomer is present in a higher content, the LCST response of the temperature sensitive component may be "eliminated" (e.g., no phase separation seen up to and above 100.degree. C.). On the other hand, graft and block copolymers of pH and temperature sensitive monomers can be synthesized which retain both pH and temperature transitions independently. Chen, G. H., and A. S. Hoffman, Nature, 373, 49-52 (1995).

Detailed Description Text (26):

Although both pendant and main chain light sensitive polymers may be synthesized and are useful compositions for the methods and applications described herein, the preferred light-sensitive polymers and copolymers thereof are typically synthesized

from vinyl monomers that contain light-sensitive pendant groups. Copolymers of these types of monomers are prepared with "normal" water-soluble comonomers such as acrylamide, and also with temperature- or pH-sensitive comonomers such as NIPAAm or AAC.

Detailed Description Text (32):

If a light-sensitive polymer is also thermally-sensitive, the UV- or visible light-stimulated conversion of a chromophore conjugated along the backbone to a more hydrophobic or hydrophilic conformation can also stimulate the dissolution or precipitation of the copolymer, depending on the polymer composition and the temperature. If the dye absorbs the light and converts it to thermal energies rather than stimulating isomerization, then the localized heating can also stimulate a phase change in a temperature-sensitive polymer such as PNIPAAm, when the system temperature is near the phase separation temperature. The ability to incorporate multiple sensitivities, such as temperature and light sensitivity, along one backbone by vinyl monomer copolymerization lends great versatility to the synthesis and properties of the responsive polymer-engineered protein conjugates. For example, dyes can be used which bind to protein recognition sites, and light-induced isomerization can cause loosening or detachment of the dye from the binding pocket (Bieth et al., Proc. Natl. Acad. Sci. USA 64:1103-1106 (1969)). This can be used for manipulating affinity processes by conjugating the dye to the free end of a temperature responsive polymer, such as ethylene oxide-propylene oxide (EO-PO) random copolymers available from Carbide. These polymers, --(CH.sub.2 CH.sub.2 O).sub.x --(CH.sub.2 --CHCH.sub.3 --O).sub.y --, have two reactive endgroups. The phase separation point can be varied over a wide range, depending on the EO/PO ratio, and one end may be derivatized with the ligand dye and the other end with an --SH reactive group, such as vinyl sulfone (VS). In this way, the VS reactive end of the polymer may be conjugated to a specific cysteine site located at a distance away from the binding pocket, such that the dye conjugated to the other end of the polymer is still able to reach and affinity bind in the pocket when the polymer conformation is in the expanded state. Then either a temperature stimulus or a combination of light and temperature stimuli may operate to remove the dye from the binding pocket, regenerating it for further affinity binding (or for enzymatic activity, if it is an enzyme). This mechanism is shown in FIG. 2c.

Detailed Description Text (33):

Random and Block Copolymers of EO/PO

Detailed Description Text (34):

Random copolymers of ethylene oxide (EO) and propylene oxide (PO) also have LCSTs or CPs ("Polyethylene Oxide," F. E. Bailey and J. V. Koleske Academic Press, NY (1976)) and have two reactive end groups, so they may be conjugated by one end to the engineered protein and other reactants, such as the light sensitive ligand depicted in FIG. 2C, may be conjugated to the other end. Temperature-sensitive block copolyethers are also available (from BASF). Triblocks of PEO-PPO-PEO are called Pluronics.TM. or poloxamers, and tetrablocks are called Tetronics.TM. or poloxamines. In the case of EO-PO random or block copolymers, a range of compositions, and molecular weights of these polymers having various reactive end groups can be obtained from Shearwater Polymers, Inc. (Huntsville, Ala.). The compositions are selected on the basis of data available on their cloud points. (BASF catalog, and "Polyethylene Oxide," F. E. Bailey and J. V. Koleske Academic Press, NY (1976)). A wider range of molecular weights of these copolyethers may be prepared than with the vinyl copolymers, since their synthesis does not use a free radical chain transfer initiation process.

Detailed Description Text (36):

The reactive end group(s) of the oligomer is (are) then derivatized with specific groups (e.g., vinyl sulfone or maleimide) which are selectively reactive with the site-specific group to be conjugated (e.g., the thiol functionality of cysteine). To introduce maleimide or vinyl sulfone groups, an amine-terminated oligomer is

preferred. The amine end group of the polymers may be conjugated with maleimide to provide thiol-reactivity (Chilkote et al., 1993). A hydroxyl-terminated polymer can be conjugated with vinyl sulfone by reaction with an excess of divinyl sulfone. The vinyl sulfone end group is typically more hydrolytically stable than the maleimide in conjugation reactions with protein thiol groups. Careful control of reactant stoichiometries and reaction conditions can yield bifunctional vinyl-type polymers such as polyNIPAAm or EO/PO random or block copolymers with different functional groups on each end of the polymer.

Detailed Description Text (38):

The term "interactive molecule" as used herein includes any molecule capable of a specific binding interaction with a target site, for example on a cell membrane, or on a molecule or atom. Thus, interactive molecules include both ligands and receptors.

Detailed Description Text (40):

The stimulus-responsive components can be conjugated to a variety of different interactive molecules, including peptides, proteins, poly- or oligo-saccharides, glycoproteins, lipids and lipoproteins, and nucleic acids, as well as synthetic organic or inorganic molecules having a defined bioactivity, such as an antibiotic or antiinflammatory agent, and which bind to a target site, for example on a molecule such as a cell membrane receptor. In one preferred embodiment the interactive molecule is a protein genetically engineered to insert a coupling site for the stimulus-responsive component at a desired site. Examples of protein interactive molecules are ligand-binding proteins, including antibodies, lectins, hormones, and receptors, and enzymes. Other molecules which bind specifically or non-specifically to a target molecule include poly- or oligosaccharides on glycoproteins which bind to receptors, for example, the carbohydrate on the ligand for the inflammatory mediators P-selectin and E-selectin, and nucleic acid sequences which bind to complementary sequences, such as ribozymes, antisense, external guide sequences for RNAase P, and aptamers.

Detailed Description Text (42):

The number of proteins whose interaction with specific binding partners can be controlled via site-specific conjugation of a stimulus-responsive component is quite large. These include, for example, antibodies (monoclonal, polyclonal, chimeric, single-chain or other recombinant forms), their protein/peptide antigens, protein/peptide hormones, streptavidin, avidin, protein A, protein G, growth factors and their respective receptors, DNA-binding proteins, cell membrane receptors, endosomal membrane receptors, nuclear membrane receptors, neuron receptors, visual receptors, and muscle cell receptors. Oligonucleotides which can be modified include DNA (genomic or cDNA), RNA, antisense, ribozymes, and external guide sequences for RNAase P, and can range in size from short oligonucleotide primers up to entire genes. Carbohydrates include tumor associated carbohydrates (e.g., Le.sup.x, sialyl Le.sup.x, Le.sup.y, and others identified as tumor associated as described in U.S. Pat. No. 4,971,905, incorporated herein by reference), carbohydrates associated with cell adhesion receptors (e.g. Phillips et al., Science 250:1130-1132 (1990)), and other specific carbohydrate binding molecules and mimetics thereof which are specific for cell membrane receptors.

Detailed Description Text (43):

In one embodiment, where the stimulus-responsive component is a polypeptide, it can be conjugated to the target interactive molecule, especially a protein, at a genetically engineered thiol attachment site, or genetically fused into the DNA sequence at an appropriate position relative to binding or active sites. (Angew. Chem. Int. Ed. Engl. 32, 819-841 (1993)).

Detailed Description Text (44):

Among the proteins, streptavidin is particularly useful as a model for other ligand-binding and substrate-binding systems described herein. Streptavidin is an

important component in many separations and diagnostic technologies which use the very strong association of the streptavidin-biotin affinity complex. (Wilchek and Bayer, Avidin-Biotin Technology, New York, Academic Press, Inc. (1990); and Green, Meth. Enzymol. 184:51-67. Protein G, a protein that binds IgG antibodies (Achari et al., Biochemistry 31:10449-10457 (1992), and Akerstrom and Bjorck, J. Biol. Chem. 261:10240-10247 (1986)) is also useful as a model system. Representative immunoaffinity molecules include engineered single chain Fv antibody (Bird et al., Science 242:423-426 (1988) and U.S. Pat. No. 4,946,778 to Ladner et al., incorporated herein by reference, Fab, Fab', and monoclonal or polyclonal antibodies. Enzymes represent another important model system, as their activity can be turned on or off or modulated by the controlled collapse of the stimulus-responsive component at the active site.

Detailed Description Text (45):

In addition to their well established uses in biotechnology, streptavidin, protein G, single-chain antibodies and enzymes are ideal model systems for several other important reasons. Genetic engineering systems for these proteins have been established, allowing convenient site-directed mutagenesis and the expression of large quantities of each protein in hosts such as E. coli. High-resolution crystal structures are available that provide a molecular "road map" of the ligand binding sites (Achari et al. supra; Hendrickson et al., Proc. Natl. Acad. Sci. USA 86:2190-2194 (1989); Weber et al., Science 243:85-88 (1992); Derrick and Wigley, Nature 359:752-754 (1992); Mian, J. Mol. Biol. 217:133-151 (1991)). This structural information provides a rational basis for the design of affinity or activity switch site-directed mutants. Of course, proteins which already have one, two or more cysteine residues located at a site convenient for attaching a stimulus-responsive component are ready for attachment of the stimulus-responsive component and need not have other cysteine residues engineered therein (unless another thiol group is desired in a specific site or useless reaction of the wild type --SH group undesirably changes the protein bioactivity). Other sites on the proteins can also be used, including amino acids substituted with non-natural amino acids.

Detailed Description Text (46):

Synthetic genes that direct the high-level expression of core streptavidin and protein G in E. coli have been constructed. See, e.g., U.S. Pat. No. 4,839,293, incorporated herein by reference. The genes incorporate biased codon usage for E. coli to maximize expression, convenient restriction sites for cassette mutagenesis, an initiating methionine, and stop codons. The synthetic genes can incorporate favored E. coli codon usage to maximize expression, and several restriction enzyme sites can be incorporated at unique sites within the gene to facilitate mutagenesis efforts. The gene sequence encoding the form of protein G for which the NMR solution structure has been reported (Gronenborn et al., Science 253:657-661 (1991)) has also been constructed. A single-chain Fv antibody gene has also been cloned and constructed from the parent antibody S5, which recognizes the CD44 receptor antigen (Sandmaier et al., Blood 76:630-635 (1990)). Single-chain antibodies consist of the variable heavy and variable light chains connected by a short peptide linker, and are constructed by using polymerase chain reaction techniques to produce a single-chain Fv antibody (Bird et al., supra; Orlandi et al., Proc. Natl. Acad. Sci. USA 86:3833-3837 (1989); and Davis et al., Bio/Technol. 9:165-169 (1991)), each of which is incorporated herein by reference.

Detailed Description Text (49):

Additionally, ion-sensitive stimulus-responsive components such as components capable of Ca^{sup.++} sensitive polymer conformational changes may be used. Additionally, single stranded DNA, one end of which is attached to a specific site on the interactive molecule, while the complementary sequence of base pairs is attached on one end of the stimulus-responsive component may be used, so that when the DNA pairs off, hooking the stimulus-responsive component to a specific site on the protein, the distance the smart stimulus-responsive component attachment is from the protein active site is controlled.

Detailed Description Text (50):

Typically the linkage site(s) will be (a) just outside the binding site of the molecule, (b) at selected distances away from the binding site, (c) just inside the binding sites, (d) deep inside the binding pockets, and/or (e) at a selected site at some distance from the binding site (f) at allosteric sites distant from the binding sites. When situated at a desired site near the binding site of the molecule, sufficient hindrance, steric or otherwise, is provided by the contracted or collapsed stimulus-responsive component to adversely affect the ligand binding equilibrium while minimally affecting the affinity of the ligand for the molecule's binding pocket or active site when the stimulus-responsive component is in the expanded state. Thus, upon stimulation, the conformational change or collapse of the polymer coil either inhibits, directly or allosterically, the entrance of the ligand to a protein binding pocket or an enzyme's active site and/or interferes with the ligand which is already bound within the site, thereby causing its complete expulsion from the binding pocket or a reduction in affinity. The polymer can also be engineered to gradually collapse, which allows the conformation of the polymer to be controlled, by gradual small, variations or changes in environment conditions, and thus the degree of reaction inhibition to be controlled over a range of intermediate affinities or reactivities. Recovery of the molecule's binding affinity or enzymatic activity can be effected by a return to the environmental conditions favoring the soluble, extended and hydrated random polymer coil. Alternatively, as shown in FIG. 2b, in some cases the hydrated coil can block the binding pocket more effectively than the collapsed coil. Additionally, there may be an advantage to using a pendant reactive group on the polymer backbone to provide better blocking of the binding site.

Detailed Description Text (52):

Sites for covalent attachment of the stimulus-responsive component on the interactive molecule may already exist or can be created by a variety of techniques depending on the nature of the molecule. For example, if the molecule is a recognition protein that already contains a cysteine residue at a desired site, e.g., in or near an enzyme's active site, this native site can be used for attachment so long as conjugation of stimulus-responsive component to any other cysteine residues that may be present in the enzyme does not adversely affect the enzyme activity or the control exerted by the stimulus-responsive component at the active site. When the molecule is a protein or nucleic acid, recombinant DNA techniques can be used to provide a site if there is not one in the native molecule suitable for linking the stimulus-responsive component. Typically, a thiol side chain (e.g., one or more cysteine residues) is genetically engineered at a predetermined surface position at or near a ligand binding site of a protein, or at a distant site such as an allosteric site (depending on the desired action of the stimulus). Techniques such as site-specific mutagenesis can be used and are described by, for example, Zoller and Smith, DNA 3:479-488 (1984), Sambrook et al., Molecular Cloning: A Laboratory Manual, 2d Ed., Cold Spring Harbor laboratory Press, Cold Spring Harbor, NY (1989), and a useful polymerase chain reaction-mediated site-directed mutagenesis procedure is described by Stappert et al., Nucleic Acid. Res. 20:624, (1992). Similar techniques can also be used to remove undesired sites that may occur in a native or wild type protein. Other reactive amino acids can also be engineered and non-natural amino acids can also serve as unique attachment sites. When the affinity molecule is an oligonucleotide, thiolated oligonucleotides for stimulus-responsive component attachment can be inserted during synthesis, as described in Ede et al., Bioconj. Chem. 5:373-376 (1994), incorporated herein by reference. With synthetic interactive molecules, unique chemical hooks such as thiols, amines, or hydroxyls can be synthetically incorporated at appropriate positions near the recognition site.

Detailed Description Text (54):

High-resolution X-ray crystallographic structures of the three proteins are used to determine appropriate positions for site-directed mutagenesis. With modeling

techniques, positions are located with appropriate solvent-accessibility for reaction with the stimulus-responsive components, and positions deeper in the binding pocket are identified which require partial unfolding for conjugation. Sites are chosen at the subunit--subunit interfaces in streptavidin and the single-chain Fv antibody to allosterically reduce the affinity by changing the protein structure by stimulating a conformational change in the polymer conjugated at a distant site. Numerous sites both around the binding sites of the three proteins and at appropriate allosteric sites away from the binding site can be used where the binding affinity will be reduced by stimulus-responsive component precipitation.

Detailed Description Text (56):

Once sites are chosen, cassette or PCR site-directed mutagenesis techniques are used to introduce cysteine residues at those positions. The engineered proteins are produced in E. coli, and then characterized, including CD characterization of secondary structure and binding affinity measurements, to confirm that the mutation itself has not adversely affected the protein structure, stability, and/or activity. The mutant proteins are then used for conjugation to the stimulus-responsive components as described below.

Detailed Description Text (57):

To facilitate placement of a stimulus-responsive component attachment site in or near the binding pocket or other desired site, site-directed binding pocket mutants can be produced to optimize the thermodynamic and kinetic properties specifically for use with stimulus-responsive components. Such mutants display altered ligand affinities and thus provide additional control over protein function for optimizing stimulus-responsive component-molecular conjugates. Libraries of site-directed affinity mutants can be used. For example, several ligand-binding site mutations have been constructed in streptavidin which alter important biotin-binding interactions, as described in copending U.S. application Ser. No. 08/387,055, incorporated herein by reference. This provides a choice for the initial ligand-binding thermodynamic properties, so that changes in affinity triggered by the stimulus-responsive component can be engineered to fall in a useful and desired range.

Detailed Description Text (58):

Depending on the stimulus-responsive component's composition, molecular weight and location of the conjugation site(s) on the molecule, environmental stimulus can be applied to affect: (a) the gradual or rapid and, if desired, reversible switching of molecular binding interactions on and off, (b) the partitioning or separation of the stimulus-responsive component-molecule conjugates (with or without their affinity targets) to selective surfaces, interfaces or second aqueous phases, and (c) a combination of (a) and (b), wherein the conjugates are first directed to a specific surface, interface, or phase, and then the bound ligand is "switched off" (released) to the local microenvironment. In case (c) the two steps may be reversed in sequence. By applying small changes in environmental stimuli, such as temperature and/or pH and/or light, to the system, one can achieve environmental control over the stimulus-responsive component-molecule microenvironment, which permits molecular level control of the recognition and binding capability of different size and composition ligands with affinity proteins or different size and composition substrates with enzymes, selectively controlling the specific bioactivity of the molecule. The change in stimulus-responsive component conformation can also expose or block the active site of an enzyme, whose action is to release a biochemical or chemical agent by enzymolysis of the agent's linkage to the smart stimulus-responsive component.

Detailed Description Text (59):

The attachment site for the stimulus-responsive components can thus be directed to well-defined positions relative to the ligand binding site of each protein. Cysteine conjugation sites can be readily engineered just outside the binding

sites, at selected distances away from the binding site, just inside the binding sites, deep inside the binding pockets, and/or at potential allosteric sites distant from the binding sites.

Detailed Description Text (60):

For example, the streptavidin Asn 49 residue lies in a solvent accessible position on the border of the biotin binding site, and this residue has been mutated to cysteine (N49C) using site directed mutagenesis techniques. Large quantities of this protein have been expressed and purified, and a vinyl sulfone-modified poly (NIPAAm) temperature-responsive stimulus-responsive component has been conjugated to this position. Streptavidin does not contain cysteine in its native sequence, making it convenient to introduce unique thiol side-chains. To design an exemplary site-directed mutant aimed at controlling its recognition processes as described herein, the general criteria center on choosing a site near the biotin binding site with significant aqueous solution exposure at the atom where the thiol is substituted. One goal is to introduce a thiol with sufficient solvent exposure (to permit relative ease of conjugation to the responsive stimulus-responsive component), and at a site where the collapse of the stimulus-responsive component should sterically interfere with ligand binding. The biotin binding site of streptavidin is covered by a loop that is extremely flexible and disordered in the ligand-free state. This loop becomes ordered and forms a "lid" over the binding site when biotin is bound. This loop represents a target for stimulus-responsive component conjugation. The site-directed mutant places a cysteine at position 49 (Asn49Cys), which borders the lid.

Detailed Description Text (61):

Like streptavidin, protein G does not contain cysteine residues in the native structure. Protein G exists naturally as a multi-domain protein with high affinity for IgG antibodies and Fc derivatives. A small (55 amino acid) domain has been genetically constructed, which retains native affinity for IgG ($K_a > 10^{10}$ M⁻¹) and which displays extremely high stability and a highly reversible folding/unfolding equilibrium (Alexander et al., Biochemistry 31:3597-3603 (1992)). Co-crystal analysis suggests that protein G recognizes IgG by forming an antiparallel interaction between its second .beta.-strand and the last .beta.-strand of the antibody heavy chain C1 domain (Derrick and Wigley, Nature 359:752-754 (1992)), while a solution NMR study suggests that the helix is involved centrally. Thus, mutants have been made that introduced unique cysteine residues near both sites. Three initial cysteine mutants have been constructed, with the thiol side-chain introduced at positions 11, 21, and 37. All three proteins have been expressed and purified in large quantities for conjugation to thiol-specific stimulus-responsive components.

Detailed Description Text (64):

Optimal solution conditions are used for site-specifically conjugating molecules to the genetically-engineered cysteines. The chemically reactive nucleophile is the thiolate anion. It is thus important to keep the side-chain reduced and to optimize the conjugation pH. The pH and the stimulus-responsive component's activated end group must also be chosen so that the competing conjugation to the amino groups of lysine side-chains is avoided. The pH will also determine the rate of hydrolysis of the thiol-specific electrophilic functionality on the stimulus-responsive component. Conjugation reactions to --SH groups are typically conducted between pH 6 and 7.5, which is below the pH where lysine amino groups are most reactive. The protein-engineered cysteine thiol groups often cross-link spontaneously in solution, so that one begins with disulfide-bonded dimers of protein G. etc. These disulfides are reduced prior to conjugation using dithiothreitol, tris(2-carboxyethyl)phosphine (TCEP), or a similar reagent. TCEP does not react with the thiol-specific electrophiles, such as the vinyl sulfones to be used here, and can thus be used directly in the conjugation solution to ensure that the thiol is kept in the active oxidation state. Typical starting reaction conditions are 50 mM phosphate buffer, pH 7.0, 100-300 .mu.M protein, 1 mM TCEP (added at least twice),

50-fold molar excess stimulus-responsive component (to protein), room temperature, 2 hours. Common variations are temperature and time (e.g. overnight at 4.degree. C.).

Detailed Description Text (66):

The conjugation mixture is then passed over a SEPHADEX.TM. G-50 gel filtration column. The stimulus-responsive component-protein conjugate is separated from the unreacted protein by thermal precipitation of the stimulus-responsive component which brings the conjugates and any unreacted stimulus-responsive component out of solution. The conjugate and any unreacted stimulus-responsive component are then redissolved in buffer and characterized by SDS-PAGE electrophoresis. The conjugate is also characterized by HPLC analysis on a GPC column, where the conjugate runs at a different molecular weight than the free protein or stimulus-responsive component. MALDI-TOFS mass spectrometry techniques can also be used to characterize the conjugate, which provides high-resolution molecular weight analysis that is strong evidence of 1:1 stoichiometry. Control experiments are conducted with wild-type protein, where there is no conjugation of the stimulus-responsive component, to confirm the specificity of the conjugation to the genetically engineered cysteine of the mutant proteins. Peptide mapping can also be conducted to exactly characterize which residue (i.e. the engineered cysteine) has been modified..

Detailed Description Text (68):

As the reactivity of engineered thiols can vary widely depending on their position on the protein surface, there are alternative reaction conditions to the basic protocol given above to increase the efficiency of the conjugation reaction. For example, the reaction efficiency is often increased greatly by adding protein denaturants at low concentrations. These denaturants unfold the protein slightly and increase the accessibility of the thiol functional groups. As outlined above, the length of reaction, temperature, and pH can also be varied to increase the efficiency of the conjugation reaction. In the above protocol, there is no separation of the unreacted stimulus-responsive component. In activity assays, a washing step can be used to remove excess stimulus-responsive component before quantitating the binding affinity. However, it is possible that the excess stimulus-responsive component would interfere somewhat with the initial binding step, and it may thus be desirable to remove the unreacted stimulus-responsive component. This is done with streptavidin and protein G by running the conjugation mixture over an affinity column. The streptavidin conjugates can be passed over a standard iminobiotin column so that the stimulus-responsive component-protein conjugate will bind, while any free stimulus-responsive component is washed through. Similarly, protein G can be passed over a commercial IgG affinity column, with the conjugate binding to the column and the stimulus-responsive component washed through. Ion-exchange chromatography can be used to separate conjugates with different stoichiometries if stimulus-responsive components have been conjugated to lysine residues in addition to the genetically engineered cysteine.

Detailed Description Text (69):

The stimulus responsive component may be coupled to the molecule using conjugation methods available in the art, and described for example, in Taylor, Ed., "Protein Immobilization", Marcel Dekker, New York, 1991; and Scouten, "Affinity Chromatography", J. Wiley & Sons, New York, 1981.

Detailed Description Text (71):

Although the foregoing description has focused on direct binding of the stimulus-responsive component to the molecule, linkers or spacer groups can also be used to connect the stimulus-responsive component to the desired site on the molecule. Nucleic acids offer the additional advantage that they can be made complementary to a target and used for targeting as well as in combination with the stimulus-responsive component to regulate access to the binding site. In a preferred embodiment, the stimulus-responsive components can be attached to a protein via an intervening oligonucleotide which is complementary in sequence to an